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(54) Title: ADENOVIRAL VECTORS

(57) Abstract

The present invention is directed to recombinant adenoviral vectors capable of highly effective therapeutic levels of p53. In particular, the present invention provides a replication competent recombinant adenovirus containing a constitutive viral or cellular promotor operably linked to a p53 gene, wherein said vector is defective in E1B55K function. The vectors of the present invention are capable of replication and lysis of neoplastic cells. The vectors may optionally include modifications to the genome so as to impart additional therapeutic or targeting functions. The present invention also provides pharmaceutical formulations of such vectors. The present invention further provides methods of use of such vectors. The present invention further provides methods of preparing the vectors.

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TITLE

ADENOVIRAL VECTORS

BACKGROUND OF THE INVENTION

In order to facilitate the understanding of the present invention, a brief overview of the life cycle of the adenovirus is offered. The adenoviral replicative cycle in human cells can be divided into the early and late phase which are punctuated by the onset of viral DNA replication. The early phase beings when viral particles attach to cells through interaction between the virion fiber domain and cell surface receptors. The virion moves into the cell by either endocytosis or direct penetration of the cytoplasmic membrane and is transported to the nucleus where most of the capsid is shed. In the nucleus, the virion core proteins are removed yielding viral chromosomes that are almost entirely devoid of virion proteins. Expression of the viral genome is temporally coordinated and begins with the E1A region about one hour after infection, The other early genes E1B, E2, E3 and E4 are expressed soon after E1A at 1.5-2.0 hours post infection. A number of the protein products encoded by the early genes are required for viral DNA replication, while other prepare the DNA synthesis machinery of the infected cell for efficient viral DNA replication. Some early virally encoded proteins have been associated with protecting infected cells from immune surveillance.

The late phase of infection with onset of DNA replication begins at about 7 hours post infection. In the native adenovirus, the messenger RNAs for all late gene products are spliced from a primary RNA which is transcribed from the major late promoter (MLP). The MLP is located at position 16.5 on the r-strand. Although the major late promoter is active to a limited extent in the early phase of infection, the transcription does not proceed past map position 39. During the late phase of the viral life cycle, the MLP is fully activated and continues to map position 99. Each late primary RNA transcript is processed into one of five different mRNAs, L1-L5. These mRNAís all contain a common tripartite leader sequence of 203 nucleotides and a common 3í end. Late mRNAs encode capsid components and proteins required for

assembly of virions and packaging of the viral chromosome. Viral DNA replication requires the terminal protein for initiation and proceed by a semiconservative mechanism. With the onset of replication, efficient transcription of the late gene families from the major late promoter begins and attains a maximal level approximately 18 hours post infection. During the late phase viral proteins block cellular DNA and protein synthesis, presumably so that maximum viral macromolecular synthesis can occur. Intermediate gene expression, which actually begins during the early phase, reaches a maximum between 8 -12 hour post infection. Assembly of the virion and packaging of the viral genome begins at about 24 hours after infection. Infected cells are killed because of attrition and lyse yielding approximately 10,000 virions per cell.

The adenovirus E1 region, which encodes the immediate early gene E1A and the early gene E1B, plays a key role in the adenovirus life cycle and is responsible for interfering with the ability of the infected cell to regulate cell cycle progression, differentiation and programmed cell death (apoptosis). The E1A gene products stimulate infected cells, which are normally differentiated and quiescent, to progress into the S-phase of the cell cycle in order that viral DNA replication can occur. Normal cells usually respond to unscheduled stimulation of cell cycle progression (by E1A or other mitogenic factors) by activation of p53-dependent apoptosis. However, in the context of a viral infection the E1A products do not stimulate apoptosis because the protein products of the E1 region gene, E1B, are effective inhibitors of apoptosis. Therefore, during the early stage of viral infection the E1A and E1B gene products cooperate to bring about a quasi-tumorigenic state in the infected cell which is required for efficient viral DNA replication and a productive infection cycle. The full scope of activity of the adenovirus E1A region is described in Bayley, S. and Mymryk, J. (1994) Intl. J. of Oncology 5:425-444. For a comprehensive review of the adenovirus biology, see Shenk, T. (1996), *Fields Virology*, 3rd Edition: p2111-2148.

The E1 genomic sequence is located at the extreme left end of the 36kb adenoviral genome. The primary E1A mRNA is differentially spliced during the early phase of replication into two prominent mRNAs, called 13S and 12S, which give rise to 289R and 243R AA proteins, respectively. The 289R and 243R proteins differ only by an internal sequence of 46 amino acids that is unique to the larger protein.

Although the E1A primary transcript is spliced into to 3 other mRNAs called 11S, 10S, and 9S, which encode for proteins of 217R, 171R and 55R respectively, these messages and not made efficiently in the early phase of infection and it is likely that the 289R and 243R proteins carry out the primary functions of E1A during the viral life cycle. The E1B gene transcript is also differentially spliced to yield mRNAs of 22S, 14.5S, 14S, and 13S each of which contains two open reading frames. One of these open-reading frames is common to all of the messages and encodes a protein of 179R. Depending on the mRNA, the other open reading frames give rise to proteins of 84R, 93R, 155R and 496R. Of the E1B proteins the 176R and 496R proteins, which are also referred to as E1B19K and E1B55K respectively, are the most prominent and best characterized.

The E1A and E1B gene products play critical roles in the productive infection cycle to prepare the infected cell for viral replication and to regulate viral specific processes. The E1A and E1B products do not contain intrinsic enzymatic activities, but are thought to carry out their functions by interacting with a number of cellular proteins. The E1A proteins associate with a wide range of cellular proteins including p400, p300, cAMP-responsive transcription binding protein (CBP), p130, p107, pRb, cyclin A, cdk2 and TATA-binding protein (TBP). Mapping studies have been used to compare cellular protein binding domains and functional domains in the E1A proteins. Stimulation of cell cycle progression by E1A has been mapped to three regions in the common amino terminal domain of the 243R and 289R proteins (Howe, *et al.*, (1990) PNAS 87:5883-5887). These regions are commonly referred to as the amino-terminal domain, conserved region 1 (CR1) and conserved region 2 (CR2). The amino-terminal domain and CR1 are required to bind a number of proteins including p300/CBP which are thought to be co-activators of gene transcription that have been implicated in regulation of cell proliferation and differentiation. The third E1A region required for cell cycle regulation by E1A is CR2 which is required for association with the know members of the pRb family of related cell cycle regulators including pRb, p107 and p130.

The pRb family members regulate the cell cycle by binding to a the E2F class of transcription factors that in turn regulate expression of genes that are required for

cell cycle phase transitions. Binding of p300 and the pRb family members appears to inactivate the ability of these proteins to suppress cell cycle progression and this appears to be the major mechanism by which E1A induces resting cells to progress into the cell cycle. A large body of evidence has accumulated to support this hypothesis. For example, by associating with pRb the E1A proteins disrupt E2F-pRb complexes which frees E2F to stimulate gene expression that allows progression into the S-phase of the cell cycle. It is not precisely known how p300 regulates cell proliferation, but p300 is known to regulate expression of genes that are required to maintain a differentiated phenotype and that inhibition of p300 can block terminal differentiation. In addition, it is known that E1A-mutants that associate with p300, but are defective for binding pRb, are nevertheless able to stimulate phosphorylation of pRb which leads to disruption of pRb-E2F complexes and cell cycle progression (Wang, *et al.*, (1991) Mol. Cell. Bio. 11, 4253-4265).

Induction of unscheduled DNA synthesis by E1A is a cellular stress that is sensed by the infected host cell. The infected cell responds by inducing apoptosis which is normally mediated by p53. The p53 gene is activated in response to a wide variety of cellular stresses including DNA damage, hypoxia and expression of mitogenic oncogenes including E1A. Productive viral infection cannot occur if the infected cell commits programmed cell death and therefore the virus has evolved to inhibit apoptosis, at least early during the infectious cycle, by production of the E1B19K and 55K products. For a review of regulation of apoptosis by E1B, see White, E. (1998) Seminars in Virology 8, 505-513. The E1B19K is considered to be the primary inhibitor of E1A-induced apoptosis because E1B19K alone blocks E1A induced apoptosis more efficiently than E1B55K alone. The E1B19K inhibits apoptosis by two different mechanisms. First, E1B19K associates with the proapoptotic Bcl-2 family members Bax, Nbk/Bik and BNIP3, and inhibit the ability of these proteins to induce apoptosis. Second, the E1B19K protein can inhibit apoptosis by interacting with factors such as FADD and CED4 which normally act to activate caspases for apoptosis. The E1B55K protein binds to and downregulates the ability of p53 to act as an activator of transcription. Therefore, the E1B55K protein can augment the E1B19K protein to inactivate p53-dependent apoptosis.

In addition to stimulating cell cycle progression and suppressing apoptotic pathways the E1A and E1B proteins also play important viral specific roles during the replication cycle of the virus. The E1A proteins initiate the coordinated expression of the viral genome by stimulating expression of promoters for the E1B gene in addition to the other early gene regions E2, E3 and E4. The 289R E1A protein is primarily responsible for the transactivation of the early adenoviral promoters and mapping studies have shown that the 46 amino acid unique domain of the 289R protein plays the major role in activation of the early viral promoters. The E1B55K protein also carries out critical viral specific roles during the productive infection cycle. Adenovirus E1B55K mutants are defective for late viral protein production and shutoff of host cell protein synthesis. Consequently these mutant viruses are defective for growth on a number of human cell lines (Babiss and Ginsberg, (1984) J.Virol. 50:202-212; Babiss *et al.*, 1985 Mol. Cell. Biol. 52552-2558; Pidler *et al.*,(1986) Mol.Cell.Biol.6:470-476; Yew *et al.*,(1990) Virology 179:795-805). More recently it has been suggested that the E1B55K may alter cell cycle controls in infected cells (Goodrum and Ornelles (1997) J. Virol. 71, 548-561) and in addition E1B55K may influence viral DNA replication (Ridgway *et al*, (1997) Virology 237:404-413).

Attempts have been made to exploit the ability of the E1B55K protein to bind p53 in the design of adenoviruses that selectively replicate in and kill p53 deficient cells by the elimination of E1B55K function. See McCormick, United States Patent No. 5,677,178 issued October 14, 1997. A particular vector, ONYX-015 contains a deletion in the E1B55K coding sequence. This prevents the expression of a E1B55K product capable of binding p53 and is claimed to result in preferential replication of the virus in p53 deficient tumor cells. However, a number of reports, in addition to data presented below, have brought the replication specificity of the E1B55K-defective viruses for p53 deficient tumor cells into question. Goodrum and Ornelles (1997) J. Virol. 71, 548-561 have suggested that the E1B55K proteins relieve growth restrictions imposed on viral replication by the cell cycle and that the ability of E1B55K mutant viruses to replicate is not mediated by the status of p53. In addition, other studies have suggested that the interaction between p53 and E1B55K may be required for efficient viral replication (Ridgway *et al.* (1997) Virology 237:404-413). Together

these observations suggest that E1B55K mutant viruses are growth defective in all cell types and do not preferentially target p53 defective tumor cells for selective cell killing.

Alternative to the idea of selectively replicating vector is the employment of a replication deficient adenoviral vector containing extensive elimination of E1 function. In particular, vectors containing elimination of E1, E2, E3 and partial E4 deletions have been employed to delivery exogenous transgenes. Such vectors have been employed to deliver the p53 gene to target cells. It has been demonstrated that the expression of an exogenously administered wild type p53 in a p53 deficient (p53 mutated or p53 null) tumor cell is capable of inducing p53-mediated apoptosis in the tumor cell. Such viral vectors for the delivery of p53 are currently under development Schering Corporation and Introgen Corporation. Again, these vectors have demonstrated acceptable toxicology profiles and therapeutic efficacy for human therapeutic applications and are in Phase II clinical trials in humans for the treatment of p53 related malignancies.

Replication deficient and selectively replicating vectors have, at least in theory, design drawbacks which are of concern to clinicians. Because the replication deficient vectors will not propagate uncontrollably in the patient, they have a more theoretically appealing safety profile. However, as effective tumor elimination requires the infection of the substantial majority of the tumor cells being infected, a substantial molar excess of vector is commonly used to insure therapeutic effectiveness. Replicating vectors are viewed as being more of an issue from a safety perspective because of their ability to replicate and potentially infect non-target (normal) cells. However, by exploiting the natural ability to the virus to propagate enables these vectors to spread to surrounding tumor cells. Since the vectors themselves are able to replicate, a lower initial dose of such vectors is required. This is favorable from an immunological perspective as well as for economic reasons in the manufacture of such agents.\

SUMMARY OF THE INVENTION

The present invention provides a replication competent recombinant adenoviral vector containing a p53 gene under control of a constituitive viral or cellular promoter wherein said vector contains deletions in the E1B55K and/or E1B19K proteins. The

invention further provides pharmaceutical formulations and methods of use of same. The present invention also provides method of making such vectors and formulations.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 are diagrammatic representations of the adenoviral vectors used in the experiments presented herein. Ad5WT represents a wild-type adenovirus type 5 genome. dl55K/CMVp53 describes a recombinant adenovirus in which the CMV-p53 expression cassette is inserted into the adenovirus type 5 genome corresponding to a deletion in nucleotides 2247-3272 of the E1B gene. E1Bdl55K describes a recombinant adenovirus which contains a deletion nucleotides 2247-3272 of the E1B gene. rAdp53 is a recombinant adenovirus containing deletions in E1 and protein IX function described in Wills, et al. (1994) Human Gene Therapy as ACN53.

Figure 2 is recovered DNA from SK-BR3 cells infected with a one hour pulse of the indicated viruses at a two different concentrations, 1.8×10^8 and 1.8×10^9 particles/ml and harvested approximately 48 hours later according to the procedures of Example 2 herein. Fragmented DNA is seen in those lanes representative of apoptosis. The figure demonstrates that the FAIC vector induces substantial apoptosis in the SK-BR3 tumor cells at both concentrations of virus.

Figure 3 is recovered DNA from NCI H358 lung non-small cell carcinoma line (p53null, pRb+) cells infected with a one hour pulse of the indicated viruses at 1.8×10^9 particles/ml and harvested approximately 48 hours later according to the procedures of Example3 herein. The characteristic smear of fragmented DNA is seen in those lanes representative of apoptosis. The figure demonstrates that the FAIC vector induces substantial apoptosis in the NCI H358 tumor cells.

Figure 4 is an agarose gel stained with ethidium bromide of a HindIII digest of recovered DNA from cells infected with the FAIC vector for a period of 48 hours according to the procedures of Example 4 herein. The upper panel represents results of experiments performed with SK-BR3 tumor cells. The lower panel contains data generated from a similar experiment performed in NCI H358 tumor cells. The results presented demonstrate that replication competent wild-type Ad5 (Ad5WT), replication competent E1Bdl55K (ZAZA) virus and replicaton competent dl55K/CMVp53 (FAIC)

virus all replicate their viral DNA well while the replication deficient adenovirus control (rAdcon) and the replication deficient vector encoding p53 (rAdp53) does not.

Figure 5 is a graphical representation of the data obtained *in vivo* in a PC-3 mouse tumor model according to the procedures of Example 5 herein. Tumor volume is plotted on the vertical axis and days following administration is plotted on the horizontal axis. As can be seen from the data presented, the virus dl55K/CMVp53 (cFAIC) was able to produce tumor regression in an *in vivo* mouse model of human cancer.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a replication competent recombinant adenovirus containing a constituitive viral or cellular promoter operably linked to a p53 gene, wherein said vector is defective in E1B55K function.

I. Replication Competent Recombinant Adenovirus:

The term "replication competent" is made in reference to a virus which is capable of replicating its genome and packaging the replicated viral genome into infectious particles in mammalian cells. It should be noted that the term "replication competent" does not generally apply to virus that can only be grown in cells which have been modified to provide deleted adenoviral functions *in trans*.

The term "recombinant adenovirus" refers to an adenovirus modified by conventional recombinant DNA technology. The term "adenovirus" is synonomous with the term "adenoviral vector" and refers to viruses of the genus adenoviridiae. The term adenoviridiae refers collectively to animal adenoviruses of the genus mastadenovirus including but no limited to human, bovine, ovine, equine, canine, porcine, murine and simian adenovirus subgenera. In particular, human adenoviruses includes the A-F sugenera as well as the individual serotypes thereof the individual serotypes and A-F subgenera including but not limited to human adenovirus types 1, 2, 3, 4, 4a, 5, 6, 7, 8, 9, 10, 11 (Ad11A and Ad 11P), 12, 13,14,15,16,17,18,19, 19a, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 34a, 35, 35p, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, and 91. The term bovine adenoviruses includes but is not limited to bovine adenovirus types 1,2,3,4,7, and 10.

The term canine adenoviruses includes but is not limited to canine types 1 (strains CLL, Glaxo, RI261, Utrect, Toronto 26-61) and 2. The term equine adenoviruses includes but is not limited to equine types 1 and 2. The term porcine adenoviruses includes but is not limited to porcine types 3 and 4. The term recombinant adenovirus also includes chimeric (or even multimeric) vectors, i.e. vectors constructed using complementary coding sequences from more than one viral subtype. See, e.g. Feng, *et al.* Nature Biotechnology 15:866-870.

In the preferred practice of the invention as exemplified herein, the recombinant adenoviral vector is derived from genus adenoviridiae. Particularly preferred viruses are derived from the human adenovirus type 2 or type 5. In the preferred practice of the invention as exemplified herein, the preferred vector is derived from the human adenoviridiae. More preferred are vectors derived from human adenovirus subgroup C. Most preferred are adenoviral vectors derived from the human adenovirus serotypes 2 and 5. The function of the E3 region may be retained, but is preferably deleted. In the most preferred practice of the invention the virus is derived human adenovirus Type 5 (wtAd5) dl309, dl327 or dl520.

II. Operably Linked:

The term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the nucleotide sequences being linked are typically contiguous. However, as enhancers generally function when seperated from the promoter by several kilobases and intronic seqences may be of variable lengths, some polynucleotide elements may be operably linked but not directly flanked and may even function in trans from a different allele or chromosome.

III. p53 Gene:

The term "p53 gene" refers to the DNA sequence encoding the wild type p53 protein, modified p53 protein, targeted p53 proteins and p53 pathway genes. The term

"wild type p53 gene" is the DNA sequence encoding the full length p53 tumor suppressor gene. The term "wild type p53 gene" includes DNA sequences encoding p53 molecules derived from human as well as other mammalian sources such as porcine p53, equine p53, bovine p53, canine p53, etc. The term p53 includes p53 molecules derived from human as well as other mammalian sources such as porcine p53, equine p53, bovine p53, canine p53, etc.

The term "modified p53 protein" refers to modifications and or deletions to the above referenced genes so as to encode functional subfragments of the wild type protein. Such modifications include deletions and/or changes in amino acid coding sequence so as to produce a protein deficient in binding to its substrate. Examples of such modified p53 proteins include modifications to p53 to increase nuclear retention, deletions such as the Δ13-19 amino acids to eliminate the calpain consensus cleavage site, modifications to the oligomerization domains (as described in Bracco, *et al.* PCT published application WO97/0492 or United States Patent No. 5,573,925). Alternatively, the p53 sequence may be modified to replace the endogenous tetramerization domains with a leucine zipper oligermization domain. Furthermore, the p53 molecule may be truncated as describe in

The term "targeted p53 protein" refers to a p53 protein or modified p53 protein which has been further modifed to introduce a targeting moiety such as a signal peptide or nuclear localization signal (NLS) to the native or modified . Examples of such targed p53 protines are fusion proteins of the wild-type or modified p53 protein with the herpes simplex virus type 1 (HSV-1) structural protein, VP22. Fusion proteins containing the VP22 signal, when synthesized in an infected cell, are exported out of the infected cell and efficiently enter surrounding non-infected cells to a diameter of approximately 16 cells wide. See, e.g.Elliott, G. & O'Hare, P. Cell. 88:223-233:1997; Marshall, A. & Castellino, A. Research News Briefs. Nature Biotechnology. 15:205:1997; O'Hare, *et al.* PCT publication WO97/05265 published February 13, 1997. A similar targeting moiety derived from the HIV Tat protein is described in Vives, *et al.* (1997) J. Biol. Chem. 272:16010-16017) and may also be employed.

IV. Constituitive Viral or Cellular Promoters:

The term "constituitive viral or cellular promoters" refers to a DNA sequence generally active in a mammalian cell which is not temporally regulated in its expression pattern. Examples of viral constituitive promoters include the cytomegalovirus immediate early (CMV) promoter, Rous Sarcoma Virus Long Terminal Repeat (RSV LTR), simian virus (SV) 40. Examples of constituitive cellular promoters include the elongation factor alpha (EF) promoter, the phospho glycerate kinase (PGK) promoter and the like.

V. Defective in E1B55K Function:

The term "defective in E1B55K function" refers to the introduction of modifications to the E1B55K gene so as to eliminate its ability to bind p53 and or inactivate the transcription stimulating activity of p53. This may include complete elimination of theE1B55K coding sequence. As previously indicated the E1B55K protein binds to p53. Consequently, in order to enhance the effect of the p53 introduced by the viral vector it is preferred to eliminate p53 binding described in McCormick, United States Patent No. 5,677,178 issued October 14, 1997, the entire teaching of which is herein incorporated by reference. In the preferred practice of the invention as exemplified herein, recombinant adenovirus contains a deletion of nucleotides 2247 to 3272 of the adenoviral genome to eliminate the function of the E1B55K protein.

VI. Demonstration of Efficacy In Vitro:

In order to demonstrate the efficacy of the vectors of the present invention, the vectors were evaluated first using *in vitro* experiments. The *in vitro* experiments presented below were designed to determine the potential of the vectors to ablate tumor cells as compared to wild type virus and several other vector constructions. The following vectors were evaluated in comparitive studies: wild type adenovirus type 5 (Ad5wt); a mutant adenovirus (dl55K/CMVp53) which contains a deletion of nucleotides 2247 to 3272 of the adenoviral genome to eliminate production of a functional E1B55K protein and contains an expression cassette with the wild type human p53 gene operably linked to the cytomegalovirus promoter (CMV) prepared in

substantial accordance with the teaching of Example 1 herein; the mutant adenovirus E1Bdl55K which contains deletion of nucleotides 2247 to 3272 of the adenoviral genome to eliminate production of a functional E1B55K protein; the replication defective adenovirus vector ACN53 (Wills, *et al.* (1994) Human Gene Therapy 5:1079-1088) in which the complete E1 region is replaced with the p53 gene under control of the CMV promoter; and a control vector which contains the E1 region deletion of ACN53 but does not encode a therapeutic transgene (ZZCB).

These vectors were assayed for their ability to replicate in and induce apoptosis in two tumor cell lines, the SK-BR3 and NCI H358 tumor cell lines. The procedures for these experiments are found in Examples 2 through 4 herein and the data is presented in Figure 2,3, and 4 of the attached drawings. As indicated by the data presented, the recombinant adenoviral vectors of the present invention are capable of replication and the induction of substantial apoptosis in tumor cell lines.

VII. Demonstration of Efficacy in Vivo:

The ability of the dl55K/CMVp53 virus to effectively eliminate tumor cells *in vitro* was confirmed in an *in vivo* animal model. The details of these experiment are contained in Example 5 herein. The results of these experiments are presented in Table 1 below and Figure 5 of the attached drawings The replication competent recombinant adenovirus expressing p53 from the CMV promoter (dl55K/CMVp53) produced tumor cell killing and elimination equivalent to wild-type adenovirus. The recombinant replication deficient adenovirus (rAdp53) did not results in tumor regression and tumor mass remained in all animals treated.

Table 1. Tumor Volume Following In Vivo Administration of cFAIC

Virus	Avg. Tumor Volume (mm^3) ± SD[1]	% T/C[2]	# Animals Tumor Free
Saline control	1266 ± 403	100	0/6
Wt Ad 5	16	1.3	5/6
E1Bdl55K	39 ± 13	3.1	3/6
dl55K/CMVp53	16	1.3	5/6
rAdp53	215 ± 93	17	0/6
rAdcon	702 ± 109	55	0/6

[1] Day 27 Post Initiation of Treatment; [2] Treated/saline control

Consequently, the in vitro data was confirmed in an in vivo model of a human prostate tumor. The foregoing results demonstrate the efficiency and superiority of the vectors of the present invention relative to previously described vectors. This data demonstrates that the vectors of the present invention are therapeutically viable for the ablation of tumor cells in vivo in mammals including human beings.

VIII. Additional Modifications to the Virus:

The present invention also provides replication competent recombinant adenoviruses containing additional modifications to the viral genome such as targeting modifications, modifications to make the vectors seletively replicating in particular cell types or phenotypic states, controlled expression characteristics, suicide genes or additional toxic elements to enhance cytotoxicity. However, this is not meant to imply that other modifications to the viral genome may not also be included.

The term "selectively replicating" refers to a recombinant viral vector capable of preferential replication in one cell type versus another cell type, in a cell in one phenotypic state relative to another phenotypic state, or in a given cell type in response to an external stimuli. Selective replication is achieved by the use of replication control elements. The term "replication control elements" refers to DNA sequences inserted into the viral genome or modifications to the viral genome in order to produce recombinant viral vectors which selectively replicate in one cell type versus another cell type, in a cell in one phenotypic state relative to another phenotypic state (cell state specific), or in a given cell type in response to an external stimuli (inducible). Examples of such replication control elements include cell-type specific promoter, cell state specific promoters, and inducible promoters.

Cell type specific replication may be achieved by the linkage of a cell type specific promoter to an early viral gene such as the E1, E1a, E2 or E4 gene when the virus is selected from the adenovirus genome. The term "cell type specific promoter" refers to promoters which are differentially activated in as a result of cell cycle progression or in different cell types. Examples of cell-type specific promoters includes cell cycle regulatory gene promoters, tissue specific promoters or pathway responsive promoters.

The term "cell cycle regulatory gene promoters" describe promoters for genes which are activated substantially upon entry into S-phase. Examples of such promoters include the E2F regulated promoters (e.g. DHFR, DNA polymerase alpha, thymidylate synthase, c-myc and b-myb promoters).

Tissue specific promoters are well known in the art and include promoters active preferentially in smooth muscle (α-actin promoter), pancreas specific (Palmiter, *et al.*(1987) Cell 50:435), liver specific Rovet, et.al. (1992) J. Biol. Chem.. 267:20765; Lemaigne, *et al.* (1993) J. Biol. Chem.. 268:19896; Nitsch, *et al.* (1993) Mol. Cell. Biol. 13:4494), stomach specific (Kovarik, *et al.* (1993) J. Biol. Chem.. 268:9917, pituitary specific (Rhodes, *et al.* (1993) Genes Dev. 7:913, prostate specific (United States Patent 5,698,443, Henderson, *et al.*issued December 16, 1997), etc.

The term "pathway-responsive promoter" refers to DNA sequences that bind a certain protein and cause nearby genes to respond transcriptionally to the binding of the protein in normal cells. Such promoters may be generated by incorporating response elements which are sequences to which transcription factors bind. Such responses are generally inductive, though there are several cases where increasing protein levels decrease transcription. Pathway-responsive promoters may be naturally occurring or synthetic. Pathway-responsive promoters are typically constructed in reference to the pathway or a functional protein which is targeted. For example, a naturally occurring p53 pathway-responsive promoter would include transcriptional control elements activated by the presence of functional p53 such as the p21 or bax promoter. Alternatively, synthetic promoters containing p53 binding sites upstream of a minimal promoter (e.g. the SV40 TATA box region) may be employed to create a synthetic pathway-responsive promoter. Synthetic pathway-responsive promoters are generally constructed from one or more copies of a sequence that matches a consensus binding motif. Such consensus DNA binding motifs can readily be determined. Such consensus sequences are generally arranged as a direct or head-to-tail repeat separated by a few base pairs. Elements that include head-to-head repeats (e.g. AGGTCATGACCT) are called palindromes or inverted repeats and those with tail-to-tail repeats are called everted repeats.

Examples of pathway-responsive promoters useful in the practice of the present

invention include synthetic insulin pathway-responsive promoters containing the consensus insulin binding sequence (Jacob, *et al.* (1995). J. Biol. Chem. 270:27773-27779), the cytokine pathway-responsive promoter, the glucocorticoid pathway-responsive promoter (Lange, *et al.*(1992) J Biol. Chem. 267:15673-80), IL1 and IL6 pathway-responsive promoters (Won K.-A and Baumann H. (1990) Mol.Cell.Biol. 10: 3965-3978), T3 pathway-responsive promoters, thyroid hormone pathway-responsive promoters containing the consensus motif: 5' AGGTCA 3.', the TPA pathway-responsive promoters (TREs), TGF–β pathway-responsive promoters (as described in Grotendorst, *et al.*(1996) Cell Growth and Differentiation 7: 469-480). Additionally, natural or synthetic E2F pathway responsive promoters may be used. An example of an E2F pathway responsive promoter is described in Parr, *et al.* (1997, Nature Medicine 3:1145-1149) which describes an E2F-1 promoter containing 4 E2F binding sites and is reportedly active in tumor cells with rapid cycling. Examples of other pathway-responsive promoters are well known in the art and can be identified in the Database of Transcription Regulatory Regions on Eukaryotic Genomes accessible through the internet at http://www.eimb.rssi.ru/TRRD.

For example, in one embodiment of the invention the vector comprises a synthetic TGF–β pathway-responsive promoter active in the presence of a functional TGF–β pathway such as the promoter containing SRE and PAI pathway responsive promoters. PAI refers to a synthetic TGF–β pathway-responsive promoter comprising sequences responsive to TGF–β signally isolated from the plasminogen activator-I promoter region. The PAI-pathway-responsive promoter may be isolated as a 749 base pair fragment isolatable from the plasmid p800luc (as described in Zonneveld, *et al.* (1988) PNAS 85:5525-5529 and available from GenBank under accession number J03836). SRE refers to a synthetic TGF–β response element comprising a repeat of 4 of the Smad-4 DNA binding sequences (GTCTAGAC as described in Zawel, *et al.* (1988) Mol. Cell 1:611-617. The SRE response element may be generated by annealing complimentary oligonucleotides encoding the Smad-4 binding sequences and cloning in plasmid pGL#3 - promoter luciferase vector (commercially available from ProMega).

Tissue specific promoters are well known in the art and include promoters active preferentially in smooth muscle (alpha-actin promoter), pancreas specific (Palmiter, *et al.* (1987) Cell 50:435), liver specific Rovet, et.al. (1992) J. Biol. Chem.. 267:20765; Lemaigne, *et al* . (1993) J. Biol. Chem. 268:19896; Nitsch, *et al* . (1993) Mol. Cell. Biol. 13:4494), stomach specific (Kovarik, *et al* . (1993) J. Biol. Chem.. 268:9917, pituitary specific (Rhodes, *et al* . (1993) Genes Dev. 7:913, prostate specific antigen promoter (United States Patent No etc.

Examples of different phenotypic states would include the neoplastic phenotype versus a normal phenotype in a given cell type. Selective replication is achieved by the use of viral replication control elements. The term viral replication control element refers to a DNA sequence engineered into the vector of the present invention such that the virus is preferentially enabled to replicate the viral genome in a particular type of target cell.

In order to achieve expression of the adenovirus in tumor cells, one may employ a tumor specific promoter to drive expression of an early gene. The term "tumor specific promoters" refers to promoters which are active in tumor cells and inactive in cells which are not transformed. Examples of tumor specific promoters include the alpha-fetoprotein promoter, the tyrosinase promoter. The use of tumor specific promoters to achieve conditional replication of adenoviral vectors is described in co-pending United States Patent Application 08/433,798 filed May 3, 1995 and International Patent Application No. PCT/US96/06199 published as International Publication No. WO 96/34969 on November 7, 1996 the entire teaching of which is herein incorporated by reference.

For example the alpha-fetoprotein promoter could be used to replace the endogenous E4 promoter and achieve greater selectivity in conditional replication . Other factors such as NF-IL6 can substitute for E1a in regulating E1a responsive promoters in the adenovirus in the absence of E1A function (Speigel,M *et al.* (1992) J. Virol 66:1021-1030) and this can be avoided by substitution of the E4 promoter with a tumor specific promoter.

Although one may use the pathway responsive promoter to drive replication of the virus in the presence of a functional pathway, alternatively, the one may use a

pathway responsive promoter to drive expression of a repressor of viral replication to control expression. The term "repressor of viral replication" refers to a protein, if expressed in a given cell, substantially represses viral replication. As will be appreciated by those of skill in the art, the repressor of viral replication will be dependent on the nature of the parent adenoviral vector from which the recombinant vector of the present invention is derived. For example, in the case of adenoviral vectors or other DNA tumor viruses, the E2F-Rb fusion construct as described in European Patent Application No. 94108445.1 published December 6, 1995 (Publication number. 0 685 493 A1) may be employed. E2F-Rb fusion protein consists of the DNA binding and DP1 heterodimerizations domains of the human E2F transcription factor protein (amino acids 95-286 of wild type E2F) fused to the Rb growth suppression domain (amino acids 379-928 of the wild type Rb protein). The E2F-Rb fusion protein is a potent repressor of E2F-dependent transcription and arrests cells in G1. The DNA binding domain is located at amino acids 128-193 and the dimerization domain is located at 194-289. The sequence of the human E2F-1 protein is available from GenBank under accession number M96577 deposited August 10, 1992. The sequences of E2F from other E2F family members of E2F from other species may be employed when constructing a vector for use in other species. In the situation where the recombinant virus is based on adenoassociated virus, the rep protein and its derivates is an effective repressor of viral replication in the absence of adenovirus infection. In the situation where the virus is derived from herpes simplex virus, the ICPO-NX, a deleted form of the immediate early protein ICPO (Liun, *et al.* (1998) J. Virol. 72:7785-7795), protein may be used as an effective repressor of viral replication. Similarly, any protein with dominant negative activity can be used as a repressor of viral replication.

The term "inducible promoter" refers to promoters which facilitate transcription of the therapeutic transgene preferable (or solely) under certain conditions and/or in response to external chemical or other stimuli. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230:426-430; Iida, *et al* . (1996) J. Virol. 70(9):6054-6059; Hwang, *et al* . (1997) J. Virol 71(9):7128-7131; Lee, *et al* . (1997) Mol. Cell. Biol.

17(9):5097-5105; and Dreher, *et al* . (1997) J. Biol. Chem. 272(46); 29364-29371. Examples of radiation inducible promoters include those induced by ionizing radiation such as the Egr-1 promoter (as described in Manome, *et al.* (1998) Human Gene Therapy 9:1409-17; Takahashi, *et al* . (1997) Human Gene Therapy 8:827-833; Joki. *et al.* Human Gene Therapy (1995) 6:1507-1513; Boothman, *et al.* (1994) volume 138 supplement pages S68-S71; and Ohno, T (1995) Tanpakushitsu Kakusan Koso 40:2624-2630), X-ray inducible promoters such as the XRE promoter (as descrbed in Boothman, *et al* . (1994) Radiation Research 138(Suppl.1):S68-S71), and UV inducible promoters such as those isolated from *Clostridium perfringens* (Garnier and Cole (1988) Mol. Microbiol. 2:607-614.

Addtionally modfications to increase the potency of the vectors of the present invention include but are not limited to alterations within E1B. The vectors of the present invention may be modified to introduce mutations in E4 to increase the cytotoxicity (Muller, *et al.* (1992) J. Virol. 66:5867-5878) or contain upregulation of viral death proteins such as E4orf4 or E3 11.6K proteins. For example, the E4 region of the adenovirus genome has been implicated in viral DNA replication, host protein synthesis shut off and viral assembly. E4orf6 is sufficient for DNA replication and late protein synthesis in immortalized cells. However, E4orf6/7 appears to be required for replication in non-dividing cells. Consequently elimination of E4orf6/7 assists in restricting the replication of the virus in immortalized (i.e. tumor cells) and may be incorporated into the vectors of the present invention.

Furthermore, E4 deletions have been shown to reduce the immongenicity of the vectors (Wang, *et al.* (1997) Gene Therapy 4:393-400; Dedieu, *et al.* (1997) J. Virol 71:4626-37), but can affec the persistence of transgene expression depending on the open reading frames of E4 retained and the promoter used to drive expresion of the transgene (Armentano, *et al.* (1997) J. Virol 71:2408-2416). The E4 region also encodes a protein (E4orf6) capable of binding to and inactivating the transciptional activity of p53. Dubner, *et al.* (1996) Science 272:1470-73). Therefore, it may be desirable to modify the E4 region to delete those open reading frames with undesriable properties for the particular virus construct while retina those with desired properties. For example, for the conditionally replication virus described

herein as the E1Bdl55K-MLP-p53, the E4 orf6 region may be deleted to reduce inactivation of p53 while retaining E4orf3 to allow continued expression of p53 and replication of the virus. In order to preserve replication competency of the virus, one of E4orf6 and E4orf3 must be retained. Examples of other E4 deleted adenoviral vectors are described in Gregory, et al United States Patent No 5,670,488 issued September 23, 1997.

The E3 region of the adenovirus encodes proteins which help adenivrally infected cells avoid clearance by the immune system (Wold, *et al.* (1995) Curr. Top. Microbiol. Immunol. 199:237-274). Upregulation of this region and subfragments thereof has been shown to prevent or decrease the immune response to virally infected cells, leading to longer term gene expression. (Ilan, *et al.* (1997) PNAS 94:2587:2592, Bunder, *et al.* (1997) J. Virol. 71:7623-28). Therefore, modifications to the E3 region (or sub-components thereof) to overexpress their proteins (e.g. by upregulating the E3 region using a strong constituitive promoter such as CMV) may be desirable to allow for a greater degree of viral replication due to its ability to avoid or delay the immune mediated clearance of infected cells.

The recombinant adenoviral vectors of the present invention may also be modifed to be defective in E1B19K function. The term "defective in E1B19K function" refers to the introduction of modifications to the E1B19K gene so as to eliminate its ability of the E1B19K protein to bind to bcl-2 family members and/or its ability to interact with FADD and CED4. This may include complete elimination of theE1B19K coding sequence. Alternatively, one may delete only portions of the E1B19K coding sequence to produce a virus defective in E1B19K function. In the preferred practice of the invention. a modification is introduced into the viral genome to delete nucleotides 1682 to 3534 to eliminate both E1B55K and E1B19K function.

The replication competent recombinant adenoviruses may also be optionally modified to possess "targeting modifications" in order to achieve preferential targeting of the virus to a particular cell type. The term "targeting modification" refers to modifications to the viral genome designed to result in preferential infectivity of a particular cell type. Cell type specificity or cell type targeting may also be achieved in vectors derived from viruses having characteristically broad infectivities such as

adenovirus by the modification of the viral envelope proteins. For example, cell targeting has been achieved with adenovirus vectors by selective modification of the viral genome knob and fiber coding sequences to achieve expression of modified knob and fiber domains having specific interaction with unique cell surface receptors. Examples of such modifications are described in Wickham, *et al.* (1997) J. Virol 71(11):8221-8229 (incorporation of RGD peptides into adenoviral fiber proteins); Arnberg, *et al.* (1997) Virology 227:239-244 (modification of adenoviral fiber genes to achieve tropism to the eye and genital tract); Harris and Lemoine (1996) TIG 12(10):400-405; Stevenson, *et al.* (1997) J. Virol. 71(6):4782-4790; Michael, *et al.*(1995) Gene Therapy 2:660-668 (incorporation of gastrin releasing peptide fragment into adenovirus fiber protein); and Ohno, *et al.* (1997) Nature Biotechnology 15:763-767 (incorporation of Protein A-IgG binding domain into Sindbis virus). Other methods of cell specific targeting have been achieved by the conjugation of antibodies or antibody fragments to the envelope proteins (see, e.g. Michael, *et al.* (1993) J. Biol. Chem. 268:6866-6869. Watkins, *et al.* (1997) gene therapy 4:1004-1012; Douglas, *et al.* (1996) Nature Biotechnology 14: 1574-1578. Alternatively, particular moieties may be conjugated to the viral surface to achieve targeting (See, e.g. Nilson, *et al.* (1996) gene therapy 3:280-286 (conjugation of EGF to retroviral proteins). These recombinantly modified vectors may be produced in accordance with the practice of the present invention.

In some instances, it may be desirable to include a suicide gene in the adenovirus. This provides a "safety valve" to the viral vector delivery system to prevent widespread infection due to the spontaneous generation of replication competent viral vectors. The term "suicide gene" refers to a nucleic acid sequence, the expression of which renders the cell susceptible to killing by external factors or causes a toxic condition in the cell. A well known example of a suicide gene is the thymidine kinase (TK) gene (see e.g. Woo, *et al.* United States Patent No. 5,631,236 issued May 20, 1997 and Freeman, *et al.* United States Patent No. 5,601,818 issued February 11, 1997) in which the cells expressing the TK gene product are susceptible to selective killing by the administration of gancyclovir.

IX. Selectively Replicating:

The term "selectively replicating" refers to a recombinant viral vector capable of preferential replication in one cell type versus another cell type (cell-type specific), in a cell in one phenotypic state relative to another phenotypic state (cell state specific), or in a given cell type in response to an external stimuli (inducible).

Cell type specific replication may be achieved by operably linking a cell type specific promoter to an early adenoviral gene such as the E1, E1A or E2 gene. The term "cell type specific promoter" refers to promoters which are differentially activated in as a result of cell cycle progression or in different cell types. Examples of cell-type specific promoters include cell cycle regulatory gene promoters, tissue specific promoters or pathway responsive promoters.

The term "cell cycle regulatory gene promoters" describe promoters for genes which are activated substantially upon entry into S-phase. Examples of such promoters include the E2F regulated promoters (e.g. DHFR, DNA polymerase alpha, thymidylate synthase, c-myc and b-myb promoters).

The term "tissue specific promoter" are promoters which are substantially more active in one tissue type versus another tissue type within the same organism. Tissue specific promoters are well known in the art and include promoters active preferentially in smooth muscle (α-actin promoter), pancreas specific (Palmiter, *et al.*(1987) Cell 50:435), liver specific Rovet, et.al. (1992) J. Biol. Chem.. 267:20765; Lemaigne, *et al.* (1993) J. Biol. Chem.. 268:19896; Nitsch, *et al.* (1993) Mol. Cell. Biol. 13:4494), stomach specific (Kovarik, *et al.* (1993) J. Biol. Chem.. 268:9917, pituitary specific (Rhodes. *et al.* (1993) Genes Dev. 7:913, prostate specific (United States Patent 5,698,443, Henderson, *et al.*issued December 16, 1997), etc.

The term "pathway-responsive promoter" refers to DNA sequences that bind a certain protein and cause nearby genes to respond transcriptionally to the binding of the protein in normal cells. Such promoters may be generated by incorporating response elements which are sequences to which transcription factors bind. Such responses are generally inductive, though there are several cases where increasing protein levels decrease transcription. Pathway-responsive promoters may be naturally occurring or

synthetic. Pathway-responsive promoters are typically constructed in reference to the pathway or a functional protein which is targeted. Synthetic pathway-responsive promoters are generally constructed from one or more copies of a sequence that matches a consensus binding motif. Such consensus DNA binding motifs can readily be determined. Such consensus sequences are generally arranged as a direct or head-to-tail repeat separated by a few base pairs.

Examples of pathway-responsive promoters useful in the practice of the present invention include synthetic insulin pathway-responsive promoters containing the consensus insulin binding sequence (Jacob, *et al.* (1995). J. Biol. Chem. 270:27773-27779), the cytokine pathway-responsive promoter, the glucocorticoid pathway-responsive promoter (Lange, *et al.*(1992) J Biol. Chem. 267:15673-80), IL1 and IL6 pathway-responsive promoters (Won K.-A and Baumann H. (1990) Mol.Cell.Biol. 10: 3965-3978), T3 pathway-responsive promoters, thyroid hormone pathway-responsive promoters containing the consensus motif: 5' AGGTCA 3.', the TPA pathway-responsive promoters (TREs), TGF–β pathway-responsive promoters (as described in Grotendorst, *et al.*(1996) Cell Growth and Differentiation 7: 469-480). Additionally, natural or synthetic E2F pathway responsive promoters may be used. An example of an E2F pathway responsive promoter is described in Parr, *et al.* (1997, Nature Medicine 3:1145-1149) which describes an E2F-1 promoter containing 4 E2F binding sites and is reportedly active in tumor cells with rapid cycling. Examples of other pathway-responsive promoters are well known in the art and can be identified in the Database of Transcription Regulatory Regions on Eukaryotic Genomes accessible through the internet at http://www.eimb.rssi.ru/TRRD.

In one embodiment of the invention, the vector comprises a synthetic TGF-b pathway-responsive promoter active in the presence of a functional TGF-β pathway such as the promoter containing SRE and PAI pathway responsive promoters. PAI refers to a synthetic TGF–β pathway-responsive promoter comprising sequences responsive to TGF–β signally isolated from the plasminogen activator-I promoter region. The PAI-pathway-responsive promoter may be isolated as a 749 base pair fragment isolatable from the plasmid p800luc (as described in Zonneveld, *et al.* (1988) PNAS 85:5525-5529 and available from GenBank under accession number J03836).

SRE refers to a synthetic TGF–β response element comprising a repeat of 4 of the Smad-4 DNA binding sequences (GTCTAGAC as described in Zawel, *et al.* (1988) Mol. Cell 1:611-617. The SRE response element may be generated by annealing complimentary oligonucleotides encoding the Smad-4 binding sequences and cloning in plasmid pGL#3 - promoter luciferase vector (commercially available from ProMega).

The use of the term "cell state specific replication" is used herein to refer to an adenovirus has been modified such that it preferentially replicates in response to a phenotypic condition within a cell. Examples of different phenotypic states may include the neoplastic phenotype versus a normal phenotype in a given cell type. Alternatively, the phenotypic state may be a rapidly dividing phenotype versus a quiescent cell.

In order to achieved selective replication of the adenovirus in rapidly dividing cells, the adenovirus may contain modifications to the E1A coding sequence so as to produce E1A gene products which are deficient in binding to one or more p300 protein family members and one or more Rb protein family member protein but retain the transactivating function of the E1A CR3 domain and a deletion of the amino acids from approximately 219 to approximately 289 of the E1A 289R protein (or approximately amino acids 173 to approximately amino acid 243 of the E1A 243R protein. In the preferred practice of the invention the deletion of the binding to the p300 family members is achieved by introducing a deletion corresponding to amino acids 4-25 of the E1A 243R and 289R proteins or amino acids 38-60 of the E1A 243R and 289R proteins. In the preferred practice of the invention the deletion of the binding to the pRb family members is achieved amino acids 111-123 of the E1A 243R and 289R proteins. Alternatively, deletion of the binding to the pRb family members may be achieved by eliminate of amino acids 124-127 of the E1A 243R and 289R proteins.

In order to achieve expression of the adenovirus in tumor cells, one may employ a tumor specific promoter to drive expression of an early gene. The use of tumor specific promoters to achieve conditional replication of adenoviral vectors is described in co-pending United States Patent Application 08/433,798 filed May 3, 1995 and International Patent Application No. PCT/US96/06199 published as

International Publication No. WO 96/34969 on November 7, 1996 the entire teaching of which is herein incorporated by reference.

A phenotypic state which may be present may be a difference in a functional pathway capable of being responded to through the use of pathway responsive promoters. Although one may use the pathway responsive promoter to drive replication of the virus in the presence of a functional pathway as described above, one may alternatively use a pathway responsive promoter to drive expression of a repressor of viral replication to control expression. The term "repressor of viral replication" refers to a protein, if expressed in a given cell, substantially represses viral replication. As will be appreciated by those of skill in the art, the repressor of viral replication will be dependent on the nature of the parent adenoviral vector from which the recombinant vector of the present invention is derived. For example, in the case of adenoviral vectors or other DNA tumor viruses, the E2F-Rb fusion construct as described in European Patent Application No. 94108445.1 published December 6, 1995 (Publication number. 0 685 493 A1) may be employed. E2F-Rb fusion protein consists of the DNA binding and DP1 heterodimerizations domains of the human E2F transcription factor protein (amino acids 95-286 of wild type E2F) fused to the Rb growth suppression domain (amino acids 379-928 of the wild type Rb protein). The E2F-Rb fusion protein is a potent repressor of E2F-dependent transcription and arrests cells in G1. The DNA binding domain is located at amino acids 128-193 and the dimerization domain is located at 194-289. The sequence of the human E2F-1 protein is available from GenBank under accession number M96577 deposited August 10, 1992. The sequences of E2F from other E2F family members of E2F from other species may be employed when constructing a vector for use in other species. In the situation where the recombinant virus is based on adenoassociated virus, the rep protein and its derivates is an effective repressor of viral replication in the absence of adenovirus infection. In the situation where the virus is derived from herpes simplex virus, the ICPO-NX, a deleted form of the immediate early protein ICPO (Liun, *et al.* (1998) J. Virol. 72:7785-7795), protein may be used as an effective repressor of viral replication. Similarly, any protein with dominant negative activity can be used as a repressor of viral replication.

One may also achieve replication of the vectors of the present invention in respone to external stimuli. For example, an early adenoviral gene may be operably linked to an inducible promoter such that the replication is permitted in reponse to the external stimuli which activates the inducible promoter. The term "inducible promoter" refers to promoters which facilitate transcription preferably (or exclusively) under certain conditions and/or in response to external chemical or other stimuli. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230:426-430; Iida, *et al.* (1996) J. Virol. 70(9):6054-6059: Hwang, *et al.* (1997) J. Virol 71(9):7128-7131; Lee, *et al.* (1997) Mol. Cell. Biol. 17(9):5097-5105; and Dreher, *et al.* (1997) J. Biol. Chem. 272(46); 29364-29371. Examples of radiation inducible promoters include those induced by ionizing radiation such as the Egr-1 promoter (as described in Manome, *et al.* (1998) Human Gene Therapy 9:1409-17; Takahashi, *et al.* (1997) Human Gene Therapy 8:827-833; Joki, *et al.* Human Gene Therapy (1995) 6:1507-1513; Boothman, *et al.* (1994) volume 138 supplement pages S68-S71; and Ohno, T (1995) Tanpakushitsu Kakusan Koso 40:2624-2630), X-ray inducible promoters such as the XRE promoter (as described in Boothman, *et al.* (1994) Radiation Research 138(Suppl.1):S68-S71), and UV inducible promoters such as those isolated from *Clostridium perfringens* (Garnier and Cole (1988) Mol. Microbiol. 2:607-614.

X. Pharmaceutical Formulations:

The present invention further provides a pharmaceutically acceptable formulation of the replication competent recombinant adenoviruses in combination with a carrier. The vectors of the present invention may be formulated for dose administration in accordance with conventional pharmaceutical practice with the addition of carriers, excipients, and/or delivery enhancing agents. Dosage formulations may include intravenous, intratumoral, intramuscular, intraperitoneal, topical, matrix or aerosol delivery formulations.

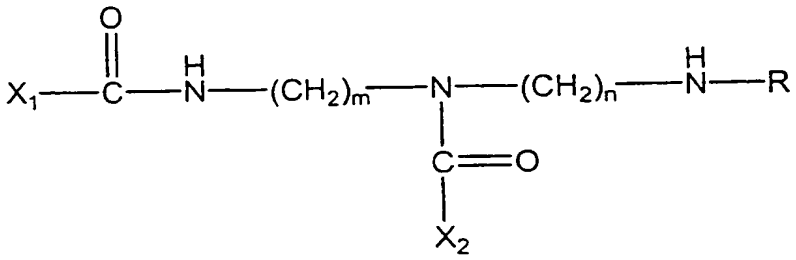
The term "carrier" refers to compounds commonly used on the formulation of pharmaceutical compounds used to enhance stability, sterility and deliverability of the therapeutic compound. When the virus is formulated as a solution or suspension, the delivery system is in an acceptable carrier, preferably an aqueous carrier. A variety of

aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorption monolaurate, triethanolamine oleate, etc.

The present invention further provides pharmaceutical formulations of the replication competent recombinant adenoviruses of the present invention with a carrier and a delivery enhancing agent(s). The terms delivery enhancers" or "delivery enhancing agents" are used interchangeably herein and includes one or more agents which facilitate uptake of the virus into the target cell. Examples of delivery enhancers are described in co-pending United States Patent Application Serial No. 09/112,074 filed July 8, 1998. Examples of such delivery enhancing agents include detergents, alcohols, glycols, surfactants, bile salts, heparin antagonists, cyclooxygenase inhibitors, hypertonic salt solutions, and acetates. Alcohols include for example the aliphatic alcohols such as ethanol, N-propanol, isopropanol, butyl alcohol, acetyl alcohol. Glycols include glycerine, propyleneglycol, polyethyleneglycol and other low molecular weight glycols such as glycerol and thioglycerol. Acetates such as acetic acid, gluconic acid, and sodium acetate are further examples of delivery-enhancing agents. Hypertonic salt solutions like 1M NaCl are also examples of delivery-enhancing agents. Examples of surfactants are sodium dodecyl sulfate (SDS) and lysolecithin, polysorbate 80, nonylphenoxypolyoxyethylene, lysophosphatidylcholine, polyethyleneglycol 400, polysorbate 80, polyoxyethylene ethers, polyglycol ether surfactants and DMSO. Bile salts such as taurocholate, sodium tauro-deoxycholate, deoxycholate, chenodesoxycholate, glycocholic acid, glycochenodeoxycholic acid and other astringents such as silver nitrate may be used. Heparin-antagonists like quaternary amines such as protamine sulfate may also be used. Cyclooxygenase

inhibitors such as sodium salicylate, salicylic acid, and non-steroidal antiinflammatory drug (NSAIDS) like indomethacin, naproxen, diclofenac may be used.

Delivery-enhancing agents includes compounds of the Formula I:



wherein n is an integer from 2-8, X1 is a cholic acid group or deoxycholic acid group, and X2 and X3 are each independently selected from the group consisting of a cholic acid group, a deoxycholic acid group, and a saccharide group. At least one of X2 and X3 is a saccharide group. The saccharide group may be selected from the group consisting of pentose monosaccharide groups, hexose monosaccharide groups, pentose-pentose disaccharide groups, hexose-hexose disaccharide groups, pentose-hexose disaccharide groups, and hexose-pentose disaccharide groups.

The term "detergent" includes anionic, cationic, zwitterionic, and nonionic detergents. Exemplary detergents include but are not limited to taurocholate, deoxycholate, taurodeoxycholate, cetylpyridium, benalkonium chloride, Zwittergent3-14 detergent, CHAPS (3-[(3-Cholamidopropyl) dimethylammoniol]-1-propanesulfonate hydrate), Big CHAP, Deoxy Big CHAP, Triton-X-100 detergent, C12E8, Octyl-B-D-Glucopyranoside, PLURONIC- F68 detergent, Tween 20 detergent, and TWEEN 80 detergent (CalBiochem Biochemicals).

Unit dosage formulations of the present invention may be included in a kit of products containing the replication competent recombinant adenovirus of the present invention lyophilized form and a solution for reconstitution of the lyophilized product along with instructions for use. Recombinant adenoviruses of the present invention may be lyophilized by conventional procedures and reconstituted.

C. Calpain Inhibitors:

Another example of a delivery enhancing agent which may be employed in the formulations of present invention include calpain inhibitors. The "calpain inhibitor" (abbreviated "CI") refers to a compound which inhibits the proteolytic action of calpain-I, e.g. μ-calpains. The term calpain inhibitors as used herein includes those compounds having calpain I inhibitory activity in addition to or independent of their other biological activities. A wide variety of compounds have been demonstrated to have activity in inhibiting the proteolytic action of calpains. Examples of calpain inhibitors are useful in the practice of the present invention include N-acetyl-leu-leu-norleucinal also known as "calpain inhibitor 1." Additional calpain inhibitors are described in the following United States Patents, herein incorporated by reference, United States Patent No. 5,716,980 entitled Alcohol or aldehyde derivatives and their use; United States Patent No. 5,714,471 entitled Peptide and peptide analog protease inhibitors; United States Patent No. 5,693,617 entitled Inhibitors of the 26s proteolytic complex and the 20s proteasome contained therein; United States Patent No. 5,691,368 entitled Substituted oxazolidine calpain and/or cathepsin B inhibitors; United States Patent No. 5,679,680 entitled.α.-substituted hydrazides having calpain inhibitory activity; United States Patent No. 5,663,294 entitled Calpain-inhibiting peptide analogs of the kininogen heavy chain; United States Patent No. 5,661,150 entitled Drug for neuroprotection; United States Patent No. 5,658,906 entitled Cysteine protease and serine protease inhibitors; United States Patent No. 5,654,146 entitled Human ice homolog; United States Patent No. 5,639,783 entitled Ketone derivatives; United States Patent No. 5,635,178 entitled Inhibition of complement mediated inflammatory response using monoclonal antibodies specific for a component forming the C56-9 complex which inhibit the platelet or endothelial cell activating function of the C56-9 complex; United States Patent No. 5,629,165 Neural calcium-activated neutral proteinase inhibitors; United States Patent No. 5,622,981 entitled Use of metabotropic receptor agonists in progressive neurodegenerative diseases; United States Patent No. 5,622,967 entitled Quinolone carboxamide Calpain inhibitors; United States Patent No. 5,621,101 entitled Protein kinase inhibitors for treatment of neurological disorders; United States Patent No. 5,554,767 entitled

Alpha-mercaptoacrylic acid derivatives having calpain inhibitory activity; United States Patent No. 5,550,108 entitled Inhibition of complement mediated inflammatory response; United States Patent No. 5,541,290 entitled Optically pure calpain inhibitor compounds; United States Patent No. 5,506,243 entitled Sulfonamide derivatives; United States Patent No. 5,498,728 entitled Derivatives of L-tryptophanal and their use as medicinals; United States Patent No. 5,498,616 entitled Cysteine protease and serine protease inhibitors; United States Patent No. 5,461,146 entitled Selected protein kinase inhibitors for the treatment of neurological disorders; United States Patent No. 5,444,042 entitled Method of treatment of neurodegeneration with calpain inhibitors; United States Patent No. 5,424,325 entitled aminoketone derivatives; United States Patent No. 5,422,359 entitled α.-aminoketone derivatives; United States Patent No. 5,416,117 entitled Cyclopropenone derivatives; United States Patent No. 5,395,958 entitled Cyclopropene derivatives; United States Patent No. 5,340,922 entitled Neural calcium-activated neutral proteinase inhibitors; United States Patent No. 5,336,783 entitled Calpain inhibitor cystamidin A and its production; United States Patent No. 5,328.909 entitled Cyclopropenone derivatives; and United States Patent No. 5,135,916 entitled Inhibition of complement mediated inflammatory response. The uses of calpain inhibitors in gene therapy protocols is further described in Atencio, *et al.*, co-pending United States Patent Applications Serial Nos. 09/172,685 and 60/104,321 filed October 15, 1998.

XI. Therapeutic Applications:

The present invention provides a method of eliminating neoplastic cells by the administration of a recombinant adenoviral vector of the present invention to said neoplastic cell. The term "neoplastic cell" is a cell displaying an aberrant growth phenotype characterized by independence of normal cellular growth controls. As neoplastic cells are not necessarily replicating at any given time point, the term neoplastic cells comprise cells which may be actively replicating or in a temporary non-replicative resting state (G1 or G0). Localized populations of neoplastic cells are referred to as neoplasms. Neoplasms may be malignant or benign. Malignant neoplasms are also referred to as cancers. The term cancer is used interchangeably

herein with the term tumor. Neoplastic transformation refers the conversion of a normal cell into a neoplastic cell, often a tumor cell.

The present invention provides a method of ablating neoplastic cells in a mammalian organism in vivo by the administration of a pharmaceutically acceptable formulation of the replication competent recombinant adenovirus described above. The term "ablating" means the substantial reduction of the population of viable neoplastic cells so as to alleviate the physiological maladictions of the presence of the neoplastic cells. The term "substantial" means a reduction in the population of viable neoplastic cells in the mammalian organism by greater than approximately 20% of the pretreatment population. The term "viable" means having the uncontrolled growth and cell cycle regulatory characteristics of a neoplastic cell. The term "viable neoplastic cell" is used hereing to distinguish said cells from neoplastic cells which are no longer capable of replication. For example, a tumor mass may remain following treatment, however the population of cells comprising the tumor mass may be dead. These dead cells have been ablated and lack the ability to replicate, even though some tumor mass may remain. The term "mammalian organism" includes, but is not limited to, humans, pigs, horses, cattle, dogs, cats.

Preferably one employs an adenoviral vector endogenous to the mammalian type being treated. However, in some instances it may be advantageous to use vectors derived from different species which possess favorable pathogenic features. For example, since humans may not have been exposed to equine or ovine adenoviruses, they may have a reduced humoral immunological response to such vectors. By minimizing the immune response, rapid systemic clearance of the vector is avoided resulting in a greater duration of action of the vector and greater efficacy of administration over a course of therapy prior to any immunological response can be mounted by the organism. For example, it is reported (WO 97/06826 published April 10, 1997) that ovine adenoviral vectors may be used in human gene therapy to minimize the immune response characteristic of human adenoviral vectors.

In the preferred practice of the invention as exemplified herein, a recombinant adenovirus containing a deletion of the E1B55K gene function and expressing the p53 gene from a constituitive promoter (e.g. CMV) is formulated with a pharmaceutically

acceptable carrier for administration by intravenous, intraperitoneal, or intratumor injection. The appropriate dose and method of administration of the vector to be administered to the mammalian organism in need of treatment will be determined by the skilled artisan taking into account the extent of metastatis of the primary tumor, the delivery enhancer(s) included in the formulation, the extent to which the immunological response is suppressed, etc. Each of these latter factors will decrease the dosage of the vector provided to the mammalian organism in need of treatment. In the preferred practice of the invention, a dosage of approximately 1×10^5 to 1×10^{13} particles (preferably 1×10^6 to 1×10^{11} particles, most preferably $\times 10^7$ to 1×10^{10} particles) will be administered to the mammalian organism in one or more doses in a treatment regimen. The typical course of treatment will be the daily administration of a pharmaceutically acceptable formulation of the vector of the present invention over a period of three to ten days, preferably five to eight days.

In a further preferred practice of the invention, the pharmaceutically acceptable carrier contains a delivery enhancing agent. In a further preferred practice of the invention, the delivery enhancing agent is a calpain inhibitor. In the most preferred practice of the invention as exemplified herein, the recombinant adenoviral vector dl55K-CMV-p53 is formulated in a carrier solution further comprising the calpain inhibitor n-acetyl-leu-leu-norcinal (calpain inhibitor 1) at a concentration of from approximately 1 to 50 micromolar. In such instances, the daily dosage may be reduced as compared to a formulation absent such delivery enhancing agents by a factor of one to two logs.

While the present invention provides a method of use of the replication competent recombinant adenoviruses alone, the replication competent recombinant adenoviruses of the present invention and formulations thereof may be employed in combination with conventional chemotherapeutic agents or treatment regimens. Examples of such chemotherapeutic agents include inhibitors of purine synthesis (e.g., pentostatin, 6-mercaptopurine, 6thioguanine, methotrexate) or pyrimidine synthesis (e.g. Pala, azarbine), the conversion of ribonucleotides to deoxyribonucleotides (e.g. hydroxyurea), inhibitors of dTMP synthesis (5-fluorouracil), DNA damaging agents (e.g. radiation, bleomycines, etoposide, teniposide, dactinomycine, daunorubicin,

doxorubicin, mitoxantrone, alkylating agents, mitomycin, cisplatin, procarbazine) as well as inhibitors of microtubule function (e.g vinca alkaloids and colchicine) and farnesyl protein transferase (FPT) inhibitors. Chemotherapeutic treatment regimens refers primarily to non-chemical procedures designed to ablate neoplastic cells such as radiation therapy.

It has been observed that the immune system is capable of recognizing and eliminating recombinant adenoviral vectors. As this would effectively reduce the quantity of adenovirus reaching the target cell, it may be advantageous to instances to administer the compounds of the present invention in combination with immunosuppressive agents such as etoposide. In the preferred practice of the invention, the immunosuppressive agent is administered in advance, preferably for about a week in advance of the introduction of the recombinant viral vector of the present invention to eliminate the humoral immune response to the viral particles. In the preferred practice of the invention, a pharmaceutically acceptable formulation of the vector of the present invention is administered intratumorally following the administration of a immunosuppressive agent for a period of from about one day to about two weeks in advance of administration of the vector. The vector is preferably an adenoviral vector and further contains a deletion of the E1B-55K protein and contains an expression cassette expressing the p53 tumor suppressor gene from the CMV promoter. In the preferred practice of the invention, the immunosuppressive agent is etoposide and is administered daily for a period of from about 1 to 7 days (preferably 3-7 days) prior to administration of the vector. In such instances, the daily dosage in the course of treatment is reduced in comparison to those dosages provided absent such immunosuppressive agents.

The present invention also provides a method of ablating neoplastic cells in a population of normal cells contaminated by said neoplastic cells ex vivo by the administration of a replication competent recombinant adenovirus of the present invention to said population. An example of the application of such a method is currently employed in ex vivo applications such as the purging of autologous stem cell products commonly known as bone marrow purging. The term "stem cell product" refers to a population of hematopoietic, progenitor and stem cells capable of

reconstituting the long term hematpoietic functionvof a patient who has received myoablative therapy. Stem cell products are conventionally obtained by apheresis of mobilized or non-mobilized peripheral blood. Apheresis is conventionally achieved through the use of known procedures using commercially available apheresis apparatus such as the COBE Spectra Apheresis System, commercially available from COBE International, 1185 Oak Street, Lakewood, CO. It is preferred that treatment conditions be optimized to achieve a "3-log purge" (i.e. removal of approximately 99.9% of the tumor cells from the stem cell produce) and most preferably a "5-log purge" (removal of approximatley 99.999% of tumor cells from the stem cell product). In the preferred practice of the invention, a stem cell product of 100 ml volume would be treated at a concentration of from about $1x10^6$ to $1x10^{10}$ particles/ml of the recombinant adenovirus of the present invention for a period of approximately 4 hours at 37°C.

The present invention provides a recombinant viral vectors capable of recruiting immature dendritic cells to a tumor site and exposing the dendritic cells to a localized high concentration of tumor antigens characteristic of the tumor present in the patient. The vectors of the present invention are specifically engineered to induce killing of tumor cells. The lysed tumor cell (or the apoptotic bodies produced by an apoptosed tumor cell) provides a rich localized concentration of tumor specific proteins. By introducing a gene encoding a dendritic cell chemoattractant, immature dendritic cells capable engulfing tumor antigens are recrutited to the site of the lysed tumor cells thereby engulfing tumor antigents and presenting these antigens to the immune system. The term "dendritic cell chemoattractants" refers to chemotactic chemokines capable of attracting and/or directing the migration of dendritic cells to a particular location. It has been demonstrated that certain chemokines, fMLP (representative of formyl peptides of bacterial origin), C5a and the C-C chemokines monocyte chemotactic protein (MCP)-3, macrophage inflammatory protein (MIP)-1 alpha/LD78, and RANTES, have been involved in the recruitment and chemotactic migration of dendritic cells. Sozzani, *et al.* (1995) J. Immunol. 1995 155(7):3292-5. Xu, *et al.* suggest that all C-C chemokines, including MCP-1, MCP-2, MCP-3, MIP1 alpha, MIP-1 beta, and RANTES, induced migration of DC-enriched cells cultured with or without IL-4. Xu, *et al.* . (1996) J.

Leukoc. Biol. 60(3):365-71. Greaves, *et al.* (1997) J. Exp. Med. 186(6):837-44, indicate that MIP-3-alpha specifically interacts with the CC chemokine receptor 6 expressed on dendritic cells capable of directing migration of dendritic cells. In the preferred practice of the invention, the dendritic cell chemoattractant is MIP-3-alpha. The dendritic cell chemoattractant may be expressed intracellular form where it is released upon cell lysis or in secreted form by the use of a signal peptide. Upon expression of the dendritic cell chemoattractant, the dendritic cells then engulf the tumor antigens or apoptotic bodies, mature and migrate through existing pathways to the lymph and present the tumor antigens to the T-cells. The resulting T-cells are then capable of recognizing and killing tumor cells. The dendritic cell chemoattractant may be expressed from a viral or cellular promoter active in the target tumor cell. The chemoattractant may also be expressed in conjunction with another gene, such as the p53 gene, from the same promoter through the use of IRES elements. See for example, Lindley, *et al.* (1994) Gene 138:165-170, or He, *et al.* (1996) Gene 172:121-125.

XII. Diagnostic Applications

In addition to therapeutic applications described above, the vectors of the present invention are also useful for diagnostic purposes. For example, the vectors of the present invention may incorporate a reporter gene in conjuction with the p53 gene. The term "reporter gene" refers to a gene whose product is capable of producing a detectable signal alone or in combination with additional elements. Examples of reporter genes includes the beta-galactosidase gene, the luciferase gene, the green fluorescent protein gene, nucleotide sequences encoding proteins detectable by imaging systems such as X-rays or magnetic field imaging systems (MRI). Alternatively, such vectors may also be employed to express a cell surface protein capable of recognition by a binding molecule such as a fluorescently labelled antibody. Examples of in vivo applications include imaging applications such as X-ray, CT scans or Magnetic Resonance Imaging (MRI).

XII. Method of Making The Compositions:

The present invention further provides a method of producing the replication competent recombinant adenovirus comprising the modifications to packaging domains described above, said method comprising the steps of:

a. infecting a producer cell with a replication competent recombinant adenovirus

b. culturing said infected producer cell under conditions so as to permit replication of the viral genome in the producer cell,

c. harvesting the producer cells, and

d. purifying the replication competent recombinant adenovirus.

The term "infecting" means exposing the replication competent recombinant adenovirus to the producer cell under conditions so as to facilitate the infection of the producer cell with the replication competent recombinant adenovirus. In cells which have been infected by multiple copies of a given virus, the activities necessary for viral replication and virion packaging are cooperative. Thus, it is preferred that conditions be adjusted such that there is a significant probability that the producer cells are multiply infected with the virus. An example of a condition which enhances the production of virus in the producer cell is an increased virus concentration in the infection phase. However, it is possible that the total number of viral infections per producer cell can be overdone, resulting in toxic effects to the cell. Consequently, one should strive to maintain the infections in the virus concentration in the range of 10^6 to 10^{10}, preferably about 10^9, virions per ml. Chemical agents may also be employed to increase the infectivity of the producer cell line. For example, the present invention provides a method to increase the infectivity of producer cell lines for viral infectivity by the inclusion of a calpain inhibitor. Examples of calpain inhibitors useful in the practice of the present invention include calpain inhibitor 1 (also known as N-acetyl-leucyl-leucyl-norleucinal, commercially available from Boehringer Mannheim). Calpain inhibitor 1 has been observed to increase the infectivity of producer cell lines to recombinant adenovirus.

The term "producer cell" means a cell capable of facilitating the replication of the viral genome of the recombinant adenovirus to be produced and capable of complementing the packaging defects of the recombinant adenovirus. A variety of mammalian cell lines are publicly available for the culture of recombinant adenoviruses. For example, the 293 cell line (Graham and Smiley (1977) J. Gen. Virol. 36:59-72) has been engineered to complement the deficiencies in E1function and is a preferred cell line for the production of the current vectors. In a similar manner, cell lines may be developed incorporating viral sequences stably integrated into the viral genome. For example, Cunningham and Davidson ((1997, Virol 197:116-124) demonstrate that overlapping cosmids may be used to complement deleted viral functions for herpes viral vectors. A similar approach may be employed to complement adenoviral and other viral elements. Additional genes, such as those encoding drug resistance, can be included to allow selection or screening for the presence of the recombinant complementing vector. Such additional genes can include, for example, genes encoding neomycin resistance, multi-drug resistance, thymidine kinase, beta-galactosidase, dihydrofolate reductase (DHFR), and chloramphenicol acetyl transferase. Examples of other producer cells parent cell lnes which may be employed include HeLa cells, PERC.6 cells (as described in publication WO/97/00326, application serial No. PCT/NL96/00244 and the A549-E1 cell line (as described in International Patent Application No. PCT/US97/810039 published February 23, 1998 as International Publication No. WO98/US3473..

The term "culturing under conditions to permit replication of the viral genome" means maintaining the conditions for the infected producer cell so as to permit the virus to propagate in the producer cell. It is desirable to control conditions so as to maximize the number of viral particles produced by each cell. Consequently it will be necessary to monitor and control reaction conditions such as temperature. dissolved oxygen, pH, etc. Commercially available bioreactors such as the CelliGen Plus Bioreactor (commercially available from New Brunswick Scientific, Inc. 44 Talmadge Road, Edison, NJ) have provisions for monitoring and maintaining such parameters. Optimization of infection and culture conditions will vary somewhat, however, conditions for the efficient replication and production of virus may be achieved by

those of skill in the art taking into considerations the known properties of the producer cell line, properties of the virus, type of bioreactor, etc. When 293 cells are employed as the producer cell line, oxygen concentration is preferably maintained from approximately 50% to approximately 120% dissolved oxygen, preferably 100% dissolved oxygen. When the concentration of viral particles (as determined by conventional methods such as HPLC using a Resource Q column) begins to plateau, the reactor is harvested.

The term "harvesting" means the collection of the cells containing the recombinant adenovirus from the media. This may be achieved by conventional methods such as diffential centrifugation or chromatographic means. At this stage, the harvested cells may be stored or further processed by lysis and purification to isolate the recombinant virus. For storage, the harvested cells should be buffered at or about physiological pH and frozen at -70C.

The term "lysis" refers to the rupture of the producer cells. Lysis may be achieved by a variety of means well known in the art. When it is desired to isolate the viral particles from the producer cells, the cells are lysed, using a variety of means well known in the art. For example, mammalian cells may be lysed under low pressure (100-200 psi differential pressure) conditions or conventional freeze thaw methods. Exogenous free DNA/RNA is removed by degradation with DNAse/RNAse.

The term "purifying" means the isolation of a substanially pure population of recombinant virus particles from the lysed producer cells. Conventional purification techniques such as chromatographic or differential density gradient centrifugation methods may be employed. In the preferred practice of the invention, the virus is purified by column chromatography in substantial accordance with the process of Huyghe *et al.* (1995) *Human Gene Therapy* 6: 1403-1416 as described in co-pending United States Patent application Serial No. 08/400,793 filed March 7, 1995.

Additional methods and procedures to optimize production of the recombinant adenoviruses of the present invention are described in co-pending United States Patent Application Serial No. 09/073,076, filed May 4, 1998.

The purified virus is then admixed with appropriate carriers or delivery enhancing agents. The solution is sterilized for individual packaging and vialed for storage.

Alternatively, the virus may be lyphophilized for storage and reconsituted in a solution containing delivery enhancing agents, buffers, preservatives, cryoprotectants and/or carriers. The lyophilized virus and the reconsitution solution may be packaged together as a kit for consumption by the end user along with instructions for appropriate handling and administration.

EXAMPLES

As will be apparent to those skilled in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed below, without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described below, are, therefore to be considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples provided below.

Example 1. Construction of dl55K/CMVp53 (cFAIC)

The E1B55K-CMV-p53 (cFAIC) adenovirus was prepared by using oligonucleotide site directed mutatgenesis technique of Deng and Nickoloff (1992) *Anal. Biochem* 200, 81-88. All of the reagents, bacterial strains, and vectors used for mutagenesis were provided in the Transformer Site-Directed mutagenesis kit (commercially availble from Clontech, Palo Alto, CA). The Ad5 region containing the sequence to be mutated was inserted into Clontech plasmid pEGFP-1 and named pXB-E1B. Two primers were designed to anneal to: (1) Ad5 sequence 2236 to 2260 and modify G2247 to thymidine and T2248 to cytosine and (2) Ad5 sequence 3255 to 3284 and modify T3272 to cytosine. A third primer was designed to eliminate the HindIII site within the original vector sequence, to provide a method for selection.

For the mutagenesis reaction, the mutagenic oligonucleotides were first phosphorylated at the 5' end, and then annealed to denatured pXB-E1B template DNA. The annealed

primer/template reactions were incubated with T4 DNA polymerase, T4 DNA ligase and deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) to synthesize a complementary mutant strand. The complementary strand synthesis reaction was then transfomed into the bacterial strain, BMH 71-18 *mutS*. This bacterial strain is defective for mis-match repair, preventing undesired repair of the mutant strand. The transformants were digested with HindIII to cut any parental plasmid strands, and retransformed into DH5α for amplification. Potential E1B mutants were then screened by restriction enzyme analysis to confirm the desired mutations.

This procedure was used to introduce restriction enzyme nuclease cleavage sites in the E1B55K coding region. The first site was introduced by modifying positions 2247 and 2248 of the wild type Ad5 genome wherein a guanine^{2247} was replaced with a thymidine and thymidine^{2248} replaced with cytosine (respectively) to introduce a EcoR1 cleavage site. This results in a modification of the E1B coding sequence at position 77 from valine to serine. A second restriction site was introduced at position 3272 wherein thymidine^{3272} was replaced with cytosine site (silent mutation) to introduce an XhoI site. The new restriction enzyme sites were used in a restriciton enzyme digest with EcoRI and Xho1.

A cassette containing the p53 coding sequence, under control of the adenovirus Major Late Promoter and tripartite leader sequence, was removed by EcoR1 and partial Xho1 digestion from the plasmid, pAd-MLP-p53. This plasmid is based on the pBR322 derivative pML2 (pBR322 deleted for base pairs 1140-2490) and contains an adenovirus type 5 sequences extending from base pair 1 to 5788 except that it is deleted for adenovirus type 5 base paris 357-3327. At ths site the Ad5 357-3327 deletion, a transciption unit is inserted which is comprised of the adenovirus type 2 major late promoter, the adenovirus type 2 tripartite leader DNA and human p53 cDNA. This EcoRI/Xho1 fragment is inserted into the EcoR1 and Xho1 sites introduced into the E1B55k coding region. The polyA sequence that follows wtAd5 pIX was amplified by PCR (Ad5 sequence 4001-4368). The primers used for amplification included sequences to introduce an EcoR1 site at the 5' end of the Ad5 sequence, and a SacII site at the 3' end of the Ad5 sequence. This fragment was then inserted into the EcoR1-SacII sites immediately following the E1B19K coding

sequence. The SacII site was included in the MLP-p53 cassette inserted previously, and was upstream of the MLP promoter sequence. The resulting E1B mutation results in a sequence encoding the first 76 amino acids of the E1B55K protein followed by 11 missense amino acids resulting in a non-functional deleted E1B protein. The CMV promoter was obtained from PCR amplification of the corresponding region of SCH58500. During PCR, a BamH1 site was introduced onto the 3' end of the CMV sequence. The MLP sequence of dl55K-MLP-p53 was then replaced by digesting with SacII and BamH1, and inserting the CMV promoter PCR fragment (with the same cohesive ends) back into the vector.

Construction of the E1B mutant/ CMV-p53 adenovirus was carried out by using homologous recombination in the adenovirus E1-region containing 293 cell line by the method of Chartier, et al. (1996) J. Virol. 70:4805-4810. This method requires two fragments of DNA, one a transfer plasmid containing the E1B55K deleted/CMV-p53 cassette and the other Ad5 viral DNA containing the wtAd5 genome ("Ad5 large fragment"), from Ad5 bp918 to the 3' ITR (bp 35935). The transfer plasmid used, pXC1-CMV53, contains wtAd5 sequences from 22-2246. For recombination to produce adenovirus cFAIC, the viral large fragment and pXC1-CMV53 were cotransfected into 293 cells by calcium phosphate mediated transfection. After 5 hours the precipitate was rinsed from the cells and normal media replaced. At 15 days after the initial transfection, viral "comets" were isolated, plaque purified two times, and subsequently viral DNA was screened using restriction enzyme analysis and DNA sequencing. Viral stocks were purified by double cesium chloride gradients and quantitated by column chromatography as described in Huyghe, *et al.* (1995) Human Gene Therapy 6:1403-1416.

Example 2. Evaluation of In Vitro Efficicacy of dl55K/CMVp53 (FAIC)

In order to demonstrate that the dl55K/CMVp53 (FAIC) virus induced apoptosis in tumor cells. SK-BR3 cells. Six well plates were seeded with 6 x 10^5 SK-BR3 cells per well. Infection was performed for a one hour pulse in a volume of 250 microliters at two different concentrations, $1.8x10^8$ particles/ml and $1.8x10^9$ particles/ml of the following vectors:

1. Mock: Non-infected cells

2. rAdcon: a recombinant adenovirus lacking E1 and protein IX function without a p53 coding sequence (Wills, et al.)
3. E1Bdl55K: A replication competent recombinant adenovirus containing the E1B-55K deletion described in Example 1 above with no exogenous transgene cassette.
4. rAd-p53: ACN53 (Wills, et al.)
5. dl55K/CMVp53: The replication competent recombinant adenovirus FAIC prepared in substantial accordance with the teaching of Example 1 above.
6. Ad5WT: Wild type adenovirus type 5.

The cells were harvested by scraping at 48 hours. The cells were resuspended in lysis buffer (10mM Tris-HCl, 5mM EDTA, 100mM NaCl, 0.05% SDS, 0.5mg/mL Proteinase K, 0.25mg/mL Pronase E) and incubated for 4 hours at 37C. The sample salt concentration was then raised to 1M by addition of 5M NaCl solution. The samples were then frozen and stored at -20°C overnight. The supernatant was clarified by centrifugation and the DNA recovered by phenol/chloroform extraction and ethanol precipation. The recovered DNA was then applied to an agarose gel and stained with EtBr. The results are presented in the Figure 2 of the attached drawings. . The characteristic smear of fragmented DNA is seen in those lanes representative of apoptosis. As can be seen from the data presented, the FAIC vector induces substantial apoptosis in the SK-BR3 tumor cells.

Example 3. Demonstration of Apoptosis in Tumor Cell Lines dl55K/CMV-p53 (FAIC)

The ability to induce apoptosis was demonstrated in a second tumor cell line, the NCI H538 cells seeded in six well plates with 1.5 x 10^6 NCI H358 cells per well. The experiment was performed in substantial accordance with the teaching of Example 2 above, except that no wild-type was included and only a single concentration (1.8 x 10^9 particles/ml) was evaluated. The results are presented in Figure 3 of the attached drawings. The characteristic smear of fragmented DNA is seen in those lanes representative of apoptosis.

Example 4. Demonstration of Replication of dl55K-MLP-p53 (FAIC)

In order to demonstrate replication of the dl55K/CMVp53 (FAIC) vector in tumor cells, SK-BR3 cells were infected in substantial accordance with the teaching of Example 2 above. However, prior to loading on the agarose gel, the recovered DNA

was subjected to a digestion with the HindIII restriction endonuclease for approximately 1 hour. The DNA was then applied to an agarose gel and stained with EtBr. The results are presented in the upper panel of Figure 3 of the attached drawings. The results demonstrate viral replication of the dl55K/CMVp53 (FAIC) construct in tumor cells. The foregoing experiment was repeated in NCI H358 lung non-small cell carcinoma line ($p53^{null}$, pRb^{+}) tumor cells in substantial accordance with the teaching of Example 3 above and the data presented in the lower panel of Figure 3 of the attached drawings. The results demonstrate viral replication of the dl55K/CMVp53 (FAIC) construct in NCI H358 tumor cells.

Example 5. Demonstration of Therapeutic Efficacy In Vivo

On day 0, 36 athymic nude-nu mice (Harlan-Sprague-Dawley, Indianapolis IN) were injected in each flank with approximately $5x10^6$ PC-3 cells (prostate carcinoma, p53 null) in 200 microliters of Dulbecco's Modified Eagle Medium (DMEM were injected subQ into flanks of nude mice. The tumors were allowed to grow for 11 days at which time they had reached a palpable size of approximately 100 mm^3. Animals were randomized by tumor size into six groups and each of 5 virus or control PBS soluton was intratumorally injected for 5 consecutive days at a dose of $1x10^{10}$ particles per injection; days 11-15 post cell injection of the PC-3 cells . Tumor volume was evaluated on days 1, 4, 7, 11, 15, 19, 23, and 27. The results of the evaluations are presented in Figure 5 of the accompanying drawings and presented in the Table 1 above.

CLAIMS

We Claim:

1. A replication competent recombinant adenovirus containing a constituitive viral or cellular promoter operably linked to a p53 gene, wherein said vector is defective in E1B55K function.
2. The adenovirus of claim 1 wherein the promoter is a constituitive viral promoter.
3. The adenovirus of claim 2 wherein the promoter is the cytomegalovirus early promoter (CMV).
4. The adenovirus of claim 3 wherein the p53 gene encodes a wild type p53 gene.
5. The adenovirus of claim 4 wherein containing a deletion of 2247 to 3272 of the E1B55K coding sequence.
6. A method of ablating neoplastic cells by the administration of replication competent recombinant adenovirus containing a constituitive viral or cellular promoter operably linked to a p53 gene, wherein said vector is defective in E1B55K function
7. The method of claim 6 wherein the promoter is a constituitive viral promoter.
8. The method of claim 7 wherein the promoter is the cytomegalovirus early promoter (CMV).
9. The method of claim 8 wherein the p53 gene encodes a wild type p53 gene.
10. The method of claim 9 wherein containing a deletion of 2247 to 3272 of the E1B55K coding sequence.
11. A pharmaceutical formulation comprising a replication competent recombinant adenovirus containing a constituitive viral or cellular promoter operably linked to a p53 gene, wherein said vector is defective in E1B55K function and a pharmaceutically acceptable carrier.
12. The formulation of claim 11 wherein the promoter is a constituitive viral promoter.
13. The formulation of claim 12 wherein the promoter is the cytomegalovirus early promoter (CMV).

14. The formulation of claim 13 wherein the p53 gene encodes a wild type p53 gene.

15. The formulation of claim 14 further comprising a calpain inhibitor.

16. The formulation of claim 15 wherein the calpain inhibitors is N-acetyl-leu-leu-norcinal.

17. The formulation of claim 16 further comprising a delivery enhancing agent selected from the group consisting of alcohols, detergents, or compounds of the formula 1.

18. A method for the elimination of cancer cells in a mammalian organism, said method comprising the administration of a pharmaceutically acceptable formulation of a replication competent recombinant adenovirus containing a constituitive viral or cellular promoter operably linked to a p53 gene, wherein said vector is defective in E1B55K function.

19. The method of claim 18 wherein the vector is administered by intraperitoneal, intravenous or intratumoral injection.

20. A kit comprising a first vial containing a sterile, lyophilized replication competent recombinant adenovirus containing a constituitive viral or cellular promoter operably linked to a p53 gene, wherein said vector is defective in E1B55K function and a second vial containing a fluid for reconsitution of said adenovirus and instructions for appropriate administration.

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(57) Abstract

The present invention is directed to recombinant adenoviral vectors capable of highly effective therapeutic levels of p53. In particular, the present invention provides a replication competent recombinant adenovirus containing a constitutive viral or cellular promotor operably linked to a p53 gene, wherein said vector is defective in E1B55K function. The vectors of the present invention are capable of replication and lysis of neoplastic cells. The vectors may optionally include modifications to the genome so as to impart additional therapeutic or targeting functions. The present invention also provides pharmaceutical formulations of such vectors. The present invention further provides methods of use of such vectors. The present invention further provides methods of preparing the vectors.

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A. CLASSIFICATION OF SUBJECT MATTER
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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